# Identification and characterization of cytosolic Hansenula polymorpha proteins belonging to the Hsp70 protein family

Titorenko, Vladimir I.; Evers, Melchior E.; Diesel, Andre; Samyn, Bart; Beeumen, Josef van; Roggenkamp, Rainer; Kiel, Jan A.K.W.; Klei, Ida J. van der; Veenhuis, Marten

*Published in:*
Yeast

*DOI:*
10.1002/(SICI)1097-0061(199607)12:9<849::AID-YEA985>3.0.CO;2-Z



*Publication date:*
1996

YEAST VOL. 12: 849–857 (1996)

# Identification and Characterization of Cytosolic *Hansenula polymorpha* Proteins Belonging to the Hsp70 Protein Family

VLADIMIR I. TITORENKO, MELCHIOR E. EVERS, ANDRE DIESEL†, BART SAMYN‡, JOSEF VAN BEEUMEN‡, RAINER ROGGENKAMP†, JAN A. K. W. KIEL, IDA J. VAN DER KLEI AND MARTEN VEENHUIS*

*Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, Kerklaan 30, 9751 NN Haren, The Netherlands*
†*Department of Microbiology, Heinrich-Heine-Universität Düsseldorf, Universitätstrasse 1, D-4000 Düsseldorf, Germany*
‡*Department of Biochemistry, Physiology and Microbiology, University of Gent, Ledeganckstraat 35, 9000 Gent, Belgium*

Received 5 December 1995; accepted 18 March 1996

We have isolated two members of the Hsp70 protein family from the yeast *Hansenula polymorpha* using affinity chromatography. Both proteins were located in the cytoplasm. One of these, designated Hsp72, was inducible in nature (e.g. by heat shock). The second protein (designated Hsc74) was constitutively present. Peptides derived from both Hsp72 and Hsc74 showed sequence homology to the cytosolic *Saccharomyces cerevisiae* Hsp70s, Ssa1p and Ssa2p. The gene encoding Hsp72 (designated *HSA1*) was cloned, sequenced and used to construct *HSA1* disruption and *HSA1* overexpression strains. Comparison of the stress tolerances of these strains with those of wild-type *H. polymorpha* revealed that *HSA1* overexpression negatively affected the tolerance of the cells to killing effects of temperature or ethanol, but enhanced the tolerance to copper and cadmium. The tolerance for other chemicals (arsenite, arsenate, $H_2O_2$) or to high osmolarity was unaffected by either deletion or overexpression of *HSA1*. The nucleotide sequence of *HSA1* was submitted to the EMBL data library and given the Accession Number Z29379.

KEY WORDS — heat shock proteins; molecular chaperones; stress tolerance; peroxisome biogenesis

## INTRODUCTION

Members of the 70 kDa heat shock (Hsp70) protein family are abundant stress proteins in many organisms (Craig *et al.*, 1993). A characteristic property of these proteins involves their ability to bind to unfolded polypeptides, which are released upon ATP binding and subsequent hydrolysis. Due to their ability to recognize and temporarily stabilize unfolded polypeptides, the Hsp70s belong to a group of proteins called molecular chaperones (Ellis and van der Vies, 1991).

Eukaryotic cells have multiple Hsp70 species, usually both heat-inducible (Hsp) and constitutive (Hsc) forms (Parsell and Lindquist, 1993). They are either cytosolic or organellar bound or may shuttle between the nucleus and the cytoplasm (Gething and Sambrook, 1992). Cytosolic Hsp70s are known to stimulate protein translocation into different organelles, whereas the organellar forms promote translocation and subsequent folding and assembly of newly-imported polypeptides (Gething and Sambrook, 1992). In addition, cytosolic Hsp70s play an important role in temperature tolerance (reviewed by Parsell and Lindquist, 1993; Welch, 1992). The role of Hsp70s in tolerance to other forms of stress (chemotolerance, osmotolerance) remains to be elucidated.

Here, we describe the identification and purification of *Hansenula polymorpha* proteins, which belong to the Hsp70 protein family. Two of these, Hsp72 and Hsc74, were shown to be cytosolic proteins. The role of the heat shock inducible Hsp72 in the tolerance of *H. polymorpha* towards various forms of stress was analysed in detail.

*Corresponding author.

CCC 0749–503X/96/090849–09

The results of these studies are presented in this paper.

## MATERIALS AND METHODS

### *Strains and growth conditions*

The *H. polymorpha* strains used in this study are NCYC495 (*leu1, ura3*) and HM1-39 (*leu1Δura3*). *Escherichia coli* strains used were: DF5αF′ (*supE44 ΔlacU169*)(ϕ*lacZM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) and JA221 (*recA1 leuB6 trpE5 hsdR*$^-$ *hsdM*$^+$). *H. polymorpha* strains were grown at the indicated temperatures on YPD (1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 1% (w/v) glucose) or in mineral medium (Veenhuis *et al.*, 1979) supplemented with 0·5% (w/v) carbon source and 0·25% (w/v) nitrogen source. Amino acids were added, when required, to a final concentration of 50 μg/ml. For solid plates 2% agar was used.

*E. coli* strains used for molecular manipulations were grown as described by Sambrook *et al.* (1989).

### *Stress treatments*

Cells were grown at 26°C to the mid-exponential growth phase. Part of the culture was maintained at 26°C, the remainder was exposed to (i) heat-shock (30 min at 43°C), (ii) various forms of chemical stress (ethanol 6% (v/v), 60 min; sodium arsenite, 0·5 mM, 30 min; sodium arsenate, 1·5 mM, 30 min; cupric sulphate, 5 mM, 30 min; cadmium chloride, 1·5 mM, 30 min; or hydrogen peroxide, 5 mM, 30 min) or (iii) osmotic stress (0·7 M-sodium chloride, 30 min).

### *Stress tolerance assays*

To measure basal tolerance to various stress conditions, cells were grown at 26°C. Equal aliquots of the cells were exposed to 56°C or treated with ethanol (20% (v/v)), sodium arsenite (50 mM), sodium arsenate (100 mM), cupric sulphate (12 mM), cadmium chloride (10 mM), hydrogen peroxide (25 mM) or sodium chloride (0·7 M). For analysis of induced tolerance to these conditions, cells were pre-incubated at 43°C or pre-treated with sub-lethal concentrations of the corresponding factors (see section on stress treatment) and subsequently exposed to lethal conditions. Samples were taken at various time intervals. The cells were washed in YPD medium before being plated in triplicate to measure colony-forming ability. In all cases plates were incubated at 26°C for 3 days before colonies were counted.

### *Cloning and sequencing of the* HSA1 *gene*

Recombinant DNA techniques were carried out using standard methods (Sambrook *et al.*, 1989). A 1·5 kb fragment was synthesized by the polymerase chain reaction (PCR) using genomic DNA of *H. polymorpha* wild-type (ATCC 34438) and two 18-mer synthetic oligonucleotides (Appligene, Heidelberg, FRG) homologous to highly conserved regions of *HSP70* genes from *Saccharomyces cerevisiae* (Mullisch *et al.*, 1986). The 1·5 kb PCR fragment was labeled by random priming with [α-$^{32}$P]dATP and used to screen a genomic DNA library of wild-type *H. polymorpha* in *E. coli* DH5αF′ by colony hybridization. The library was constructed in pHARS1 (Didion and Roggenkamp, 1992). Southern analysis of plasmid DNA obtained from a positive clone revealed that a 3·4 kb *Bam*HI fragment of the genomic insert contained a complete hsp70 gene. This fragment was subcloned into Bluescript M13+ (Short *et al.*, 1988) resulting in plasmid pKSp-70. Nested unidirectional deletions were generated using the double-stranded Nested Deletion Kit (Pharmacia, Uppsala, Sweden) and sequenced by the dideoxy-chain-termination method (Sanger *et al.*, 1977) using the T7 Sequencing Kit (Pharmacia, Uppsala, Sweden) and [α-$^{35}$S]dATP.

### *Disruption and overexpression of* HSA1

For disruption of *HSA1* a plasmid was constructed in which a 1·36 kb *Bgl*II-*Nde*I fragment of pKSp-70 (containing 1·3 kb of the *HSA1* coding region including the ATG) was replaced by a 2·47 kb *Bam*HI-*Nde*I fragment of the *LEU2* gene of *S. cerevisiae*. The disrupted *HSA1* gene (*hsa1::LEU2*) was separated from vector sequences with appropriate restriction enzymes and introduced into *H. polymorpha* HM1-39 (*leu1 Δura3*) (Dohmen *et al.*, 1991). Leu$^+$ prototrophs were screened by colony hybridization using the deleted *HSA1* fragment as a probe. In a selected positive colony disruption of the *HSA1* gene was confirmed by Southern analysis.

To construct an *HSA1* overexpression strain, plasmid pHSA1 was constructed by cloning a 6·2 kb *Hin*dIII genomic fragment containing the *HSA1* gene including 3·0 kb of the 5′ flanking region into plasmid Yip5 (Struhl *et al.*, 1979). The *hsa1* disruption mutant (Δ*hsa1*) was transformed

with pHSA1. Southern analysis, using the *HSA1* fragment which has been deleted in Δ*hsa1* as a probe, revealed multi-copy integration of pHSA1.

*Analytical procedures*

Crude extracts were prepared as described before (Waterham *et al.*, 1992). Protein concentrations were determined with the BioRad protein assay kit using bovine serum albumin as standard. Proteins from crude extracts or subcellular fractions were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) and subjected to Western blotting (Kyhse-Andersen, 1984). Western blots were decorated using polyclonal antibodies against mitochondrial Hsp70 (Ssc1p), monoclonal antibodies against Hsp70 of bovine brain (Sigma, St Louis, U.S.A.) or polyclonal antibodies against Hsp72 of *H. polymorpha*.

For micro-sequencing, proteins were electroblotted on nitrocellulose and digested with endoproteinase Lys-C according to Fernandez *et al.* (1992). After extraction the peptides were separated on an RPLC column (C2-C18, 3·2 × 20 mm, 3 μm) using a SMART system (Pharmacia, Uppsala, Sweden). The peptides were eluted with a gradient of 0·05% TFA in water to 0·04% TFA in 70% acetonitrile. N-terminal sequence analysis was performed on a 476A pulsed liquid sequenator, equipped with an on-line PTH-amino acid analyser (Applied Biosystems, Foster City, CA).

Affinity chromatography using immobilized denatured proteins was carried out as described by Evers *et al.* (1993).

*Subcellular fractionation studies*

Protoplasts of wild-type *H. polymorpha* were homogenized and subjected to differential centrifugation (5 min at 500 *g*, 30 min at 30 000 *g* and 3 h at 230 000 *g*) (Douma *et al.*, 1985). The pellets were resuspended in equal volumes of homogenization buffer. Equal volumes of each fraction were used for Western blotting. In addition the 30 000 *g* pellets were subjected to sucrose density centrifugation. Peroxisomal and mitochondrial peak fractions were identified by the activities of the marker proteins alcohol oxidase (peroxisomes) and cytochrome *c* oxidase (mitochondria) (Douma *et al.*, 1985).

*Electron microscopy*

Samples were prepared for electron microscopy by established procedures (Waterham *et al.*, 1992). Immunolabelling was performed on ultrathin sections of Unicryl embedded whole cells using gold-conjugated goat-anti-rabbit antibodies (Amersham, U.K.) as described by the manufacturer.

## RESULTS

*Purification and characterization of the Hsp70 proteins from* H. polymorpha

Using affinity chromatography, based on the binding of proteins to unfolded polypeptides and the subsequent release by Mg-ATP, molecular chaperones can be identified and purified from crude cell extracts (Evers *et al.*, 1993). Using this method, three putative Hsp70s (with molecular masses of 70, 72 and 74 kDa respectively) were purified from methanol-grown cells of *H. polymorpha*, which had been subjected to heat shock.

As shown in Figure 1, the 70 kDa protein was recognized by antibodies against mitochondrial Hsp70 (Ssc1p) of *S. cerevisiae*. We previously showed that this protein, designated Hsc70, represents the mitochondrial Hsp70 of *H. polymorpha* (Evers *et al.*, 1993). Monoclonal antibodies against bovine brain Hsp70 specifically recognized the 74 kDa protein, designated Hsc74. The 72 kDa protein, called Hsp72, was recognized by none of the available antisera. Polyclonal antibodies, raised against *H. polymorpha* Hsp72, were highly specific for Hsp72 and showed no cross-reaction with either Hsc70 of Hsc74 (Figure 1).

In order to establish whether Hsp72 and Hsc74 represent *H. polymorpha* molecular chaperones, we studied their properties in binding to denatured proteins (Evers *et al.*, 1993). Hsp72 and Hsc74 were able to bind to all denatured, immobilized proteins tested (alcohol oxidase, amine oxidase, bovine serum albumin, horseradish peroxidase) and could subsequently be eluted by ATP/$Mg^{2+}$; however, both proteins failed to bind to the native form of these proteins (data not shown).

Next, we micro-sequenced various peptides derived from Hsp72 and Hsc74. The sequences showed high identity to corresponding fragments of Ssa1p and Ssa2p, cytosolic Hsp70s of *S. cerevisiae* (Table 1).

The subcellular location of Hsp72 and Hsc74 was determined by subcellular fractionation (Figure 1B). Western blot analysis revealed that both Hsp72 and Hsc74 were exclusively present in the soluble fractions and therefore represent cytosolic proteins of the Hsp70 protein family.

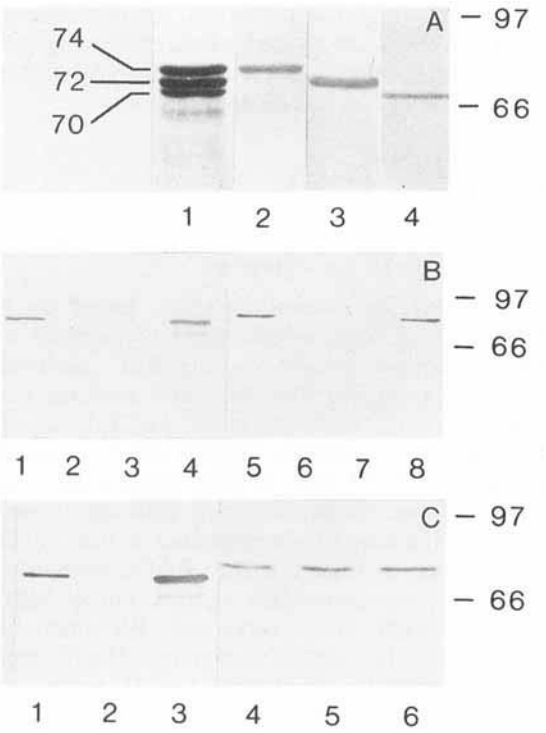


Figure 1. (A) Identification of Hsp70 proteins from wild-type *H. polymorpha*. Proteins of crude extracts of heat-shocked methanol-grown *H. polymorpha* cells were eluted from an immobilized alcohol oxidase-containing column by Mg-ATP. Proteins were separated by SDS–PAGE and stained with Coomassie Brilliant Blue (lane 1) or subjected to Western blotting. The blots were decorated with antibodies against Hsp70 of bovine brain (lane 2), Hsp72 of *H. polymorpha* (lane 3) or against the mitochondrial Hsp70 of *S. cerevisiae* (lane 4). (B) Subcellular localization of Hsp72 and Hsc74. Equal portions of subcellular fractions were used for Western blot analysis using antibodies against Hsp72 (lanes 1–4) or Hsc74 (lanes 5–8). Hsp72 and Hsc74 were present in the 500 *g* supernatant (lanes 1, 5) and the 230 000 *g* supernatant (lanes 4, 8) but not in the 30 000 *g* pellet (lanes 2, 6) or the 230 000 *g* pellet (lanes 3, 7). (C) Western blot analysis using crude extracts prepared from wild-type *H. polymorpha* (lanes 1, 4), the Δ*hsa1* (lanes 2, 5) and the *HSA* overexpression strain (lanes 3, 6) strain. Cells were grown at 30°C on methanol. Western blots were decorated with antibodies against Hsp72 (lanes 1–3) or Hsc74 (lanes 4–6).

The analysis of peroxisomal and mitochondrial peak fractions confirmed the presence of Hsc70 in mitochondria. However, in the peroxisomal peak fractions, Hsps could not be detected with the affinity purification procedure described above.

*Hsp72 is inducible and involved in stress tolerance*

We examined the expression levels of Hsp72 and Hsc74 in variously-grown *H. polymorpha* cells as well as in cells exposed to high temperature or chemical/osmotic stress. As shown in Figures 2 and 3, the level of Hsc74 was approximately constant and independent from growth substrate, temperature and chemical/osmotic stress. In contrast, Hsp72 was not detectable in glucose-grown cells at 26°C and induced during growth of cells on glycerol, ethanol and methanol at this temperature (Figure 2A, lanes 1–4). A similar pattern was observed when cells were grown at 43°C (Figure 2B, lanes 1–4). A strong induction of Hsp72 was observed when cells, grown at 26°C, were exposed to heat shock. The induction by heat shock was independent from the carbon source used for growth. Hsp72 was also significantly induced by chemical and—to a lower extent—osmotic stress (Figure 3).

In order to obtain further insight into the function of Hsp72, we cloned and sequenced the gene encoding Hsp72, *HSA1*. The nucleotide sequence was submitted to the EMBL data library and given the accession number Z29379. The deduced amino acid sequence of the cloned gene showed high similarity to the *S. cerevisiae* Ssa1p (83·3% identity) and Ssa2p (83·1% identity; Table 2).

On Western blots prepared from crude extracts of the Δ*hsa1* disruption mutant, Hsp72 was not detectable, while it was present at enhanced levels in the *HSA1* overexpression strain as compared to wild-type *H. polymorpha* (Figure 1C). These data confirm that *HSA1* encodes *H. polymorpha* Hsp72.

To examine the role of Hsp72 in the stress tolerance of *H. polymorpha*, we compared the stress tolerance of wild-type cells and cells from the Δ*hsa1* and the Hsp72-overproducing strain. For determination of the basal tolerance to killing conditions by high temperature or high ethanol concentrations, cells grow in batch cultures at 26°C on methanol were shifted to 56°C or exposed to 20% ethanol. In Hsp72-overproducing cells the basal tolerance to both stress conditions was significantly reduced, while the Δ*hsa1* mutant showed an enhanced tolerance (Figure 4A, C). A mild pretreatment of cells (43°C for 30 min or 6% ethanol for 60 min) drastically increased the tolerance to both forms of stress in both wild-type and Δ*hsa1* cells; this effect was less evident in cells overexpressing *HSA1* (Figure 4B, D). Thus, Hsp72 overproduction negatively affects the tolerance of *H. polymorpha* to killing effects of temperature or ethanol.

Table 1. Amino acid sequences of peptides derived from *H. polymorpha* (A) Hsp72 and (B) Hsc74 and alignment with *S. cerevisiae* Ssa1p

| | | | | |
|---|---|---|---|---|
| (A) | 4 | 137 | 158 | 221 |
| Hsp72 | AVGIDLG | VTDAVIT | DAGLIAG | ATAGDTH |
| | \|\|\|\|\|\|\| | \|\|\|·\| | \|\|\|\|\|\| | \|\|\|\|\|\| |
| Ssa1p | AVGIDLG | VNDAVVT | DAGTIAG | ATAGDTH |
| | 4 | 137 | 158 | 218 |
| | 329 | 362 | 388 | 425 |
| Hsp72 | SQVAEIV | SINPDEA | TQDLLLL | SEIFSTY |
| | \|\|\|\|\|\| | \|\|\|\|\|\| | \|\|\|\|\|\| | \|\|\|\|\|\| |
| Ssa1p | SQVDEIV | SINPDEA | TQDLLLL | FEIFSTY |
| | 326 | 359 | 385 | 422 |
| | 573 | 609 | | |
| Hsp72 | AIEETIS | DLYAAGG | | |
| | \|\|\|\|\| | \|\|\|\|\| | | |
| Ssa1p | KAEETIS | KLYQAGG | | |
| | 568 | 604 | | |

| | | | |
|---|---|---|---|
| (B) | | | |
| Hsc74 | AVXIDLGTTY | NQAXMNPAN | FDDPEVQNDXK |
| | \|\|\|\|\|\|\|\|\| | \|\|\|\|\|\|\|·\| | \|\|\|\|\|\|\|\|\|\| |
| Ssa1p | AVGIDLGTTY | NQAAMNPHN | FDDPEVTNDAK |
| | 4 | 55 | 76 |
| Hsc74 | PXIQVEFK | LVSDFFNGK | TQDLXLXXTA |
| | \|\|\|\|\|\|\| | \|\|\|\|\|\|\|\|\| | \|\|\|\|\|\|\|\|\|\| |
| Ssa1p | PQIQVEFK | LVSDFFNGK | TQDLLLLDVA |
| | 99 | 347 | 385 |
| Hsc74 | LIPRNTTIPXK | GRLSQELIN | NTLESYAYS |
| | \|\|\|\|\|·\|\|\|\| | \|\|\|\|\|\|\|\|\| | \|\|\|\|\|\|\|\|\| |
| Ssa1p | LIPRNSTIPTK | GRLSKEDIE | NQLESIAYS |
| | 410 | 505 | 537 |

Identical amino acids are indicated by (|), similar ones by (·). The numbers represent the number of the amino acid in the complete protein (NB: for Hsc74 these numbers are not known yet). X, not identified.

In contrast, the basal and induced tolerance of the cells to copper and cadmium was negatively affected by disruption of *HSA1*, whereas the tolerance was increased in cells overexpressing *HSA1* (Figure 4E–H).

Analysis of the basal and induced tolerance to sodium arsenite, sodium arsenate, hydrogen peroxide and high osmolarity revealed no significant differences between the wild type and both mutant strains (data not shown).

*Peroxisome biogenesis does not depend on Hsp72*

The induction of Hsp72 under conditions that require peroxisomal enzymes for growth (e.g. methanol) may point to a role of this cytosolic chaperone in the biogenesis of these organelles. This prompted us to study the subcellular location of various peroxisomal matrix proteins in the Δ*hsa1* strain grown on methanol at 30°C, 37°C or 43°C. However, both fractionation experiments

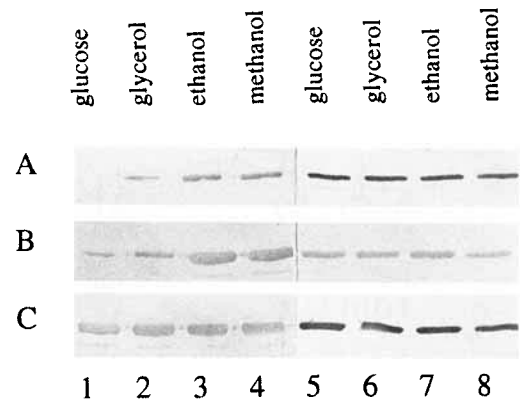


Figure 2. Protein levels of Hsp72 and Hsc74 in crude extracts prepared from wild-type *H. polymorpha*. Cells were grown at 26°C (A) or 43°C (B) in mineral media supplemented with the indicated carbon sources and harvested at the mid-exponential growth phase. In addition, cultures grown at 26°C were subjected to a heat shock (30 min at 43°C; C). Equal amounts of protein were subjected to SDS–PAGE and Western blotting. The blots were decorated with antibodies against Hsp72 (lanes 1–4) or Hsc74 (lanes 5–8).

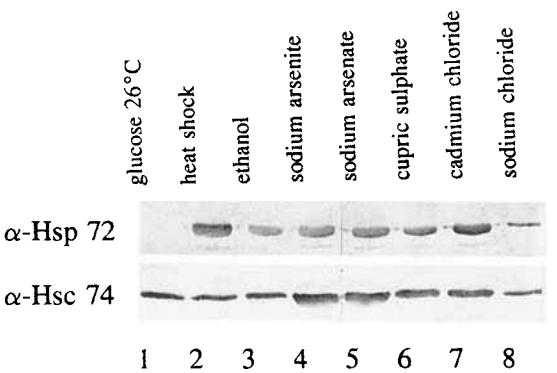


Figure 3. Effect of different forms of stress on the levels of Hsp72 and Hsc74. *H. polymorpha* wild-type cells were grown at 26°C to the mid-exponential growth phase on glucose and maintained at 26°C (lane 1; control), or exposed to various stress conditions (lanes 2–8; for details see Materials and Methods). Crude extracts were subjected to SDS–PAGE and Western blotting. The blots were decorated with antibodies against Hsp72 (upper panel) or Hsp74 (lower panel).

and immunocytochemistry revealed no differences in subcellular distribution of the three major peroxisomal matrix proteins (alcohol oxidase, dihydroxyacetone synthase and catalase) in methanol grown mutant and wild-type cells; invariably these enzymes were confined to peroxisomes (data not shown). Thus, peroxisome biogenesis in *H. polymorpha* is not hampered by deletion of *HSA1*.

Table 2. Pairwise similarity scores between the gene products of the *H. polymorpha HSA1*, *HSA2*, and the *S. cerevisiae SSA1*, *SSA2*, *SSA3* and *SSA4* genes. The numbers represent the percentage of identical amino acid residues between the primary sequences

| | HSA1 | HSA2 | SSA1 | SSA2 | SSA3 | SSA4 |
|---|---|---|---|---|---|---|
| HSA1 | — | | | | | |
| HSA2 | 89·9 | — | | | | |
| SSA1 | 83·3 | 83·6 | — | | | |
| SSA2 | 83·1 | 83·9 | 97·7 | — | | |
| SSA3 | 79·9 | 78·6 | 79·7 | 79·4 | — | |
| SSA4 | 80·9 | 80·9 | 81·6 | 81·5 | 86·9 | — |

## DISCUSSION

This paper describes the purification and characterization of two cytosolic proteins from the yeast *H. polymorpha*, designated Hsp72 and Hsc74. Based on their functional and structural properties (e.g. the binding to denatured proteins and amino acid sequence homologies), we conclude that both proteins belong to the Hsp70 protein family.

The gene encoding Hsp72 (*HSA1*) was cloned and sequenced. It shows high sequence homology to both Ssa1p and Ssa2p, cytosolic Hsp70s from *S. cerevisiae* (Table 2). Micro-sequencing of peptides obtained from Hsc74 revealed that this protein is highly homologous to the *S. cerevisiae* Ssa proteins as well. The sequence information available so far is too limited to conclude to which of the Ssa proteins (Ssa1–4) highest homology exists. Recently, a second gene encoding a *H. polymorpha* Hsp70 has been cloned and sequenced (A. Diesel and R. Roggenkamp, unpublished results). This gene, designated *HSA2* (accession number U49932), is highly homologous to *HSA1* (89·9% identity; Table 2). Most likely this gene encodes a second cytosolic Hsp70 of *H. polymorpha*; akin to the protein product of *HSA1*, it does not contain an N-terminal extension, characteristic for mitochondrial/endoplasmic reticulum (ER) Hsp70s and also lacks a C-terminal ER-retention signal. However, based on the data available so far we cannot yet conclude whether the protein product of *HSA2* is Hsc74.

Unexpectedly, in *H. polymorpha* overexpression of *HSA1* (encoding Hsp72) led to an increased sensitivity of the cells towards high temperatures and high ethanol concentrations. Apparently, Hsp72 does not play a role in the protection of

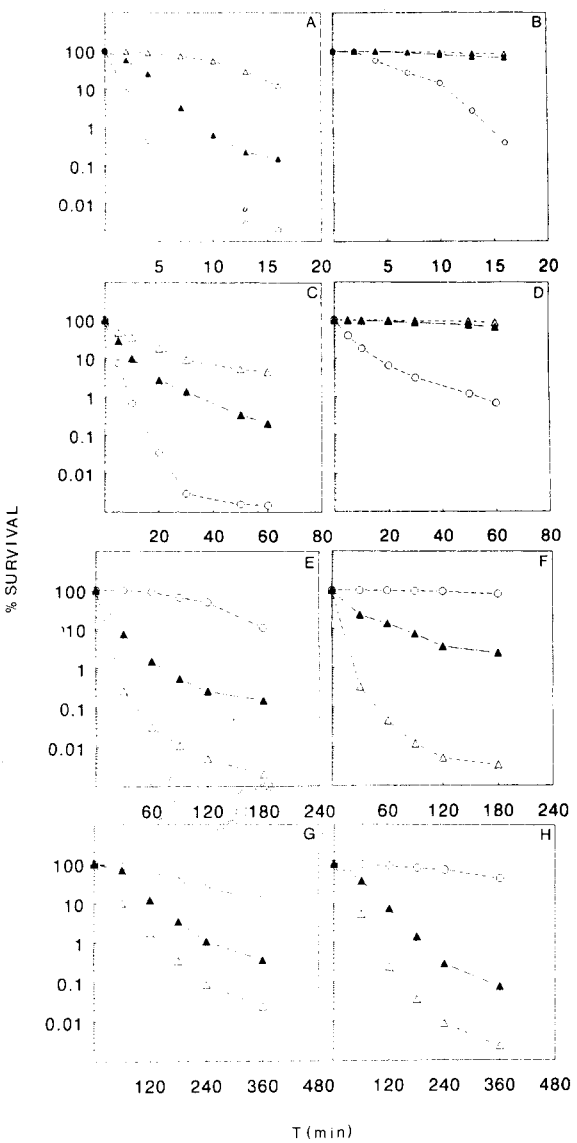


Figure 4. Survival (%) of the wild-type strain (▲), the Δ*HSA* disruption strain (Δ) and the *HSA* overexpression strain (○) after exposure of cells to various stress conditions. Cells from the mid-exponential growth phase on methanol (26°C) were exposed to 56°C without (A) or after (B) pretreatment at 43°C for 30 min, exposed to 20% ethanol without (C) or with (D) pretreatment with 6% ethanol for 60 min, exposed to 12 mM-cupric sulphate without (E) or after (F) pretreatment with 5 mM-cupric sulphate for 30 min or exposed to 10 mM-cadmium chloride without (G) or after (H) pretreatment with 1·5 mM-cadmium chloride for 30 min.

the cells against these stress factors. A possible explanation for this finding is that the increased levels of Hsp72 result in the repression of the synthesis of other, so far unidentified, heat shock proteins specifically involved in heat and ethanol tolerance.

Hsp72 is, however, essential to protect *H. polymorpha* cells from lethal lesions produced by copper and cadmium. The tolerance to other harmful compounds like arsenite, arsenate and hydrogen peroxide and also the osmotolerance was unaffected by disruption or overexpression of *HSA1*.

Previous studies on *S. cerevisiae* revealed that in this organism different stress conditions cause different modes of protein damage which are recognized by different systems for protein protection and repair. Hsp104 for instance is of critical importance in tolerance to extreme temperatures and ethanol, but less important to protect the cells from chemical stress (e.g. exerted by arsenite, copper or cadmium; Sanchez *et al.*, 1992).

Our data led us to conclude that also in *H. polymorpha* various (at least three) mechanisms exist which function in stress tolerance. One of these (heat and ethanol tolerance) is negatively affected by increased Hsp72 levels in the cells, conditions which positively affect the second mechanism (protection against copper and cadmium). A third mechanism is independent from levels of Hsp72 (e.g. osmotolerance and protection against arsenate and arsenite).

Besides their role in stress tolerance, cytosolic Hsp70s are also involved in the import of cytosolically synthesized proteins into mitochondria and the ER. In *S. cerevisiae* this was shown using a conditional mutant in which the synthesis of cytosolic Hsp70s could be controlled; when the synthesis was shut off, mitochondrial precursor proteins and proteins destined for the secretory pathway accumulated in the cytosol (Deshaies *et al.*, 1988).

We have not found evidence for an indispensable role of the methanol-inducible Hsp72 of *H. polymorpha* in peroxisomal protein import, since the development of these organelles was virtually unaffected in Δ*hsa1* cells. These results are in line with the observations of Walton *et al.* (1994) who showed that in mammalian cells the constitutive Hsp73, but not the inducible Hsp72 is involved in peroxisomal protein import.

However, because in Δ*hsa1* we are not able to completely deplete the cytosol from Hsp70 proteins, we cannot exclude a role of this class of proteins (e.g. Hsc74) in peroxisomal protein import.

Moreover, the presence of 'classical' hsps inside peroxisomes could not be demonstrated. This implies that either such proteins are indeed not present and that peroxisomes contain a different class of molecular chaperones or that the assembly of peroxisomal matrix proteins is either spontaneous or takes place outside the organelle. Folded, active proteins can be imported into peroxisomes (Glover *et al.*, 1994; McNew and Goodman, 1994), which supports the latter assumption.

## ACKNOWLEDGEMENTS

We thank Jan Zagers for skilful technical assistance. R.R. is supported by the Deutsche Forschungsgemeinschaft (Ro662/3-1) and J.V.B. by a Flemish Concerted Research Action grant (12052293).

## REFERENCES

Craig, E. A., Gambill, B. D. and Nelson, R. J. (1993). Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol. Rev.* **57,** 402–414.

Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* **332,** 800–809.

Didion, T. and Roggenkamp, R. (1992). Targeting signal of the peroxisomal catalase in the methylotrophic yeast *Hansenula polymorpha*. *FEBS Lett.* **303,** 113–116.

Dohmen, J. R., Strasser, A. W. M., Hohner, C. B. and Hollenberg, C. P. (1991). An efficient transformation procedure enabling long term storage of competent cells of various yeast genera. *Yeast* **7,** 691–692.

Douma, A. C., Veenhuis, M., de Koning, W., Evers, M. E. and Harder, W. (1985). Dihydroxyacetone synthase is localized in the peroxisomal matrix of methanol-grown *Hansenula polymorpha*. *Arch. Microbiol.* **143,** 237–243.

Ellis, R. J. and Van der Vies, S. M. (1991). Molecular chaperones. *Annu. Rev. Biochem.* **60,** 321–347.

Evers, M. E., Huhse, B., Titorenko, V. I. *et al.* (1993). Affinity purification of molecular chaperones of the yeast *Hansenula polymorpha* using immobilized denatured alcohol oxidase. *FEBS Lett.* **321,** 32–36.

Fernandez, J., De Mott, M., Atherton, D. and Mische, S. M. (1992). Internal protein sequence analysis: enzymatic digestion for less than 10 μg of protein bound to polyvinylidene difluoride or nitrocellulose. *Anal. Biochem.* **201,** 255–264.

Gething, M.-J. and Sambrook, J. (1992). Protein folding in the cell. *Nature* **355,** 33–45.

Glover, J. R., Andrews, D. W. and Rachubinski, R. A. (1994). *Saccharomyces cerevisiae* proximal thiolase is imported as a dimer. *Proc. Natl. Acad. Sci. USA* **91,** 10541–10545.

Kyhse-Andersen, J. (1984). Electroblotting of multiple gels: a simple apparatus without buffertank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Meth.* **10,** 203–209.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

McNew, J. A. and Goodman, J. M. (1994). An oligomeric protein is imported into peroxisomes *in vivo*. *J. Cell Biol.* **127,** 1245–1257.

Mullisch, K., Fallona, F., Scharf, S., Saiki, R., Horn, G. and Ehrlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* **51,** 263–273.

Parsell, D. A. and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27,** 437–496.

Sambrook, J., Fritsch, E. G. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanchez, Y., Parsell, D. A., Taulien, J., Vogel, J. L., Craig, E. A. and Lindquist, S. (1993). Genetic evidence for a functional relationship between Hsp104 and Hsp70. *J. Bacteriol.* **175,** 6484–6491.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequence analysis with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

Short, J. M., Fernandez, J. M., Sorge, J. A. and Huse, W. D. (1988). Lambda ZAP: A bacteriophage lambda expression vector with *in vivo* excision properties. *Nucl. Acids Res.* **16,** 75–83.

Struhl, K., Stinchcomb, D. T., Scherer, S. and Davis, R. W. (1979). High frequency transformation of yeast: autonomous replication of hybrid DNA molecules. *Proc. Natl. Acad. Sci USA* **76,** 1035–1039.

Titorenko, V. I., Waterham, H. R., Cregg, J. M., Harder, W. and Veenhuis, M. (1993). Peroxisome biogenesis in the yeast *Hansenula polymorpha* is controlled by a complex set of interacting gene products. *Proc. Natl,. Acad. Sci USA* **90,** 7470–7474.

Veenhuis, M., Keizer, I. and Harder, W. (1979). Characterization of peroxisomes in glucose-grown *Hansenula polymorpha* and their development after the transfer of cells into methanol-containing media. *Arch. Microbiol.* **120,** 167–175.

Walton, P. A., Wendland, M., Subramani, S., Rachubinski, R. A. and Welch, W. J. (1994). Involvement of 70-kD heat-shock proteins in peroxisomal import. *J. Cell Biol.* **125,** 1037–1046.

Waterham, H. R., Keizer-Gunnink, I., Goodman, J. M., Harder, W. and Veenhuis, M. (1992). Development of multipurpose peroxisomes in *Candida boidinii* grown in oleic acid-methanol limited continuous cultures. *J. Bacteriol.* **174,** 4057–4063.

Welch, W. J. (1992). Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. *Physiol. Rev.* **72,** 1063–1081.